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AUTHORITY

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AD_____

Award Number: DAMD17-97-1-7341

TITLE: The Use of NF1 and NF2 Mutant Mouse Strains in the Investigation of Gene Function and Disease Development

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REPORT DATE: October 1999

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 1999	Annual (30 Sep 98 - 29 Sep 99)

4. TITLE AND SUBTITLE
The Use of NF1 and NF2 Mutant Mouse Strains in the Investigation of Gene Function and Disease Development

5. FUNDING NUMBERS
DAMD17-97-1-7341

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Massachusetts Institute of Technology
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
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12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***
Mouse strain carrying germline mutations in the Nf1 and Nf2 tumor suppressor genes have been used as mouse models of human neurofibromatosis types 1 and 2, and cells derived from these mice have been used to address the normal functions of the Nf1 and Nf2 genes. Progress over the past year has included the completion and publication of two mouse models of NF1: a chimeric model which develops benign neurofibromas and a combined germline Nf1/p53 mutant model that develops MPNSTs. Genetic modifier screens using the Nf1/p53 model have proceeded to the point of identifying strain-specific differences in tumor latency and tumor spectrum in some strain combinations. Predisposition to astrocytoma in these mice was also discovered in the course of this analysis. Biochemical analysis of the Nf1 gene product, neurofibromin, has shown that is rapidly degraded following mitogenic stimulation, in a PKC-dependent manner. This process appears to limit the strength and duration of the Ras pathway signaling. Cell biological studies have implicated Nf2 in the regulation of the Rac1 pathway, and Rac1 also stimulates the phosphorylation of the Nf2 product, merlin. Progress is reported toward the isolation of the Rac1-stimulated merlin kinase. Finally, conditional expression systems are being developed to express Nf2 in a number cell types to assess its function in growth control, tumor suppression and cell survival.

14. SUBJECT TERMS
neurofibromatosis, NF1, NF2, mouse models, gene targeting, signal

15. NUMBER OF PAGES 24

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Limited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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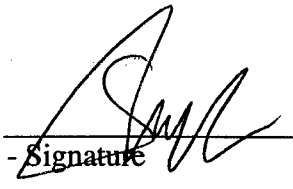

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Introduction

Neurofibromatosis type I and II (NF1 and NF2) are human genetic diseases affecting the nervous system. In both diseases, individuals inherit a loss-of-function mutation in a tumor suppressor gene and are thereby predisposed to the development of a characteristic set of lesions. Although not demonstrated experimentally in every case, it is believed that the development of these lesions in both NF1 and NF2 is dependent on the somatic mutation of the remaining wild-type allele of the relevant tumor suppressor gene. The genes responsible for NF1 and NF2 were cloned in 1990 and 1993, respectively (1-5). The *NF1* encodes a 2818 amino acid protein (neurofibromin) which contains a domain that is structurally and functionally related to the mammalian p120GAP protein as well as the IRA proteins of Saccharomyces cerevisiae (6,7). Structurally unrelated to *NF1*, the *NF2* gene encodes a protein (merlin) that belongs to a family of cytoplasmic proteins believed to link transmembrane proteins to the cytoskeleton (4,5). Merlin is most similar to a group of three closely-related proteins: ezrin, radixin and moesin (the ERM proteins).

This grant supports research in my laboratory directed at the investigation of the normal functions of the *NF1* and *NF2* genes and their roles in disease development. The focus of this research is a series of mouse strains carrying germline mutations in the Nf1 and Nf2 genes. We have previously shown that these mice are cancer prone, but fail to develop the hallmark symptoms of the human diseases (8,9). Second generation models have been produced or are under development to address the limitations of these models in an attempt to understand these species-specific differences. Cells derived from embryos homozygous for these mutations have been used to begin to characterize the normal functions of neurofibromin and merlin. Finally, a series of biochemical, cell biological, and genetic studies are in progress, that collectively address the regulation and function of these two important human disease genes.

Body

This progress report has been divided into sections corresponding to the Technical Objectives/Specific Aims of the original grant, which was initiated on 30 September, 1997.

1. Development of existing mouse models of NF1. As detailed in the previous annual report, we have constructed two second-generation models of human NF1, each displaying different features of the human disease. The development and characterization of the *Nf1*+/+:*Nf1*-/- chimera model of benign neurofibroma formation as well as the *Nf1/p53* "cis" model of malignant peripheral nerve sheath tumors (MPNSTs) was recently published (10) and will not be described further here. The use of the *Nf1/p53* strain in a genetic modifier screen and our recent discovery of a brain tumor phenotype in these mice is discussed below. Although the chimera model was quite important in addressing the difference in the phenotype between *NF1/Nf1* mutant humans and mice and does provide a possible avenue for further analysis of benign neurofibroma formation or treatment, it is a difficult model with which to work. This is due primarily to the low frequency of chimera generation upon injection of *Nf1*-/- embryonic stem (ES) cells into

blastocysts. Therefore, we do not plan to pursue this model further at this time. Instead, we have contacted Luis Parada (UT, Dallas), who has made a conditional loss-of-function allele of *Nf1*. In collaboration with the Parada laboratory, we plan to develop mouse models of neurofibroma formation through somatic inactivation of *Nf1* using the avian retrovirus delivery system developed by Varmus and co-workers (11). These experiments are not expected to begin until July, 2000.

2. Structure/function analysis of neurofibromin. As discussed above, the protein encoded by the *NF1* gene, neurofibromin, exhibits similarity to the catalytic domains of mammalian and yeast Ras-GAP proteins and has been shown to stimulate the GTPase activity of Ras *in vitro*. Importantly, *NF1*-deficient tumors exhibit elevated levels of Ras-GTP supporting the notion that deregulation of Ras activity contributes to the development of these lesions. However, while this connection to the Ras pathway is well established, nothing is known about how neurofibromin is regulated or in what biological contexts it normally functions to inhibit Ras activity. Our goal here has been to elucidate these regulatory mechanisms and define how they are specifically involved in attenuating Ras-dependent signaling events. Results from these studies should lead to a better understanding of the biological consequences of *NF1* mutations and will serve as a foundation for *in vitro* structure/function studies and *in vivo* analysis using the YAC based approach described previously. We have begun this analysis by examining neurofibromin expression in response to mitogenic stimuli. Serum deprivation resulted in an increase in neurofibromin protein levels as compared to cycling cells (data not shown). However, as shown in Figure 1, re-exposure to serum caused degradation of neurofibromin . This process was rapid and transient; degradation occurred in less than 2 minutes and the protein returned to untreated levels by 30 minutes after serum addition. Importantly, neurofibromin degradation occurred in several cell types in response to a variety of growth factors (including LPA, PDGF, EGF, bFGF; data not shown). Examination of the neurofibromin amino acid sequence revealed a putative PEST sequence, commonly found in proteins regulated by ubiquitin-mediated degradation. To examine whether the proteasome was indeed involved in regulating neurofibromin protein levels, proteasome inhibitors were tested for their ability to inhibit degradation and were found to inhibit growth factor induced neurofibromin degradation. (Fig. 2) By introducing into cells a construct encoding a his-tagged ubiquitin molecule, we were able to show that neurofibromin is indeed a direct target of the ubiquitination machinery (Fig. 2).

Because phosphorylation-induced ubiquitination and degradation is a common paradigm of regulation (e.g. I kappa B, beta-catenin), we scanned the neurofibromin sequence for putative phosphorylation sites and found several potential PKC phosphorylation sites. We therefore tested whether PKC might be involved in regulating neurofibromin levels and found that PKC inhibitors blocked its degradation. (Fig. 3) PMA, which potently activates classical and novel PKC enzymes also induced degradation, demonstrating that activation of PKC is sufficient for this response (data not shown). Consistent with this observation, activated PKC enzymes also resulted in neurofibromin down-regulation when transfected into cells. Finally, we have found that PKC enzymes can phosphorylate neurofibromin *in vitro* (Fig. 3). We are currently mapping this phosphorylation site and determining whether this phosphorylation event is involved in triggering neurofibromin ubiquitination.

To investigate how this regulation might effect the mitogenic response, we have examined the growth properties of wild-type versus *Nf1*-deficient mouse embryonic fibroblasts (MEFs). We had previously shown that under normal growth conditions (e.g. 10% serum), we found no difference in the rate of proliferation of *Nf1*+/+ versus *Nf1* -/- cells; however, *Nf1*–deficient cells were consistently more sensitive to low concentrations of serum and growth factors (see previous annual report). More recently, we have examined the activity of MAP kinase, which is a marker of the Ras pathway. As expected, there was no difference in the amplitude or duration of MAP kinase activation in response to stimulation with 10% serum (Fig. 4). However, in response to low concentrations of serum (0.2-1%), MAP kinase activity was only transiently activated in wild-type cells but was sustained in *Nf1*-deficient cells. Importantly, the duration of MAP kinase activity has been shown to be critical in sending a specific proliferative or differentiative response in a variety of cell systems. In the MEF system, the duration of MAP kinase activity correlated precisely with the mitogenic dose of serum required in cells of both genotypes. Finally, we and others have shown that Nf1-deficient cells are resistant to certain inducers of apoptosis (12 and our unpublished data; see previous annual report). Given the results described above, these data might be explained by constitutively higher levels of Ras signaling in Nf1 mutant cells leading to the increased activity of the pro-survival kinase AKT/PKB. Indeed, the activation state of AKT is higher in *Nf1*-/- MEFs compared to wild-type MEFs in low serum growth conditions (data not shown).

Taken together, these studies demonstrate that neurofibromin is dynamically regulated by the proteasome, in response to a number of growth factors and in a PKC-dependent manner. Furthermore, the MEF studies suggest that the upregulation of neurofibromin is critical for limiting the Ras, MAP kinase and AKT pathways in the presence of limiting concentrations of growth factors. These findings may be related to the development of NF1-related symptoms, in particular neurofibromas, which may develop due to an increased sensitivity to growth factors in the nerve, leading to inappropriate cell proliferation and/or cell survival. The identification and confirmation of regulatory sites within the protein (i.e. the PEST sequence, the PKC phosphorylation sites) will allow us to test this hypothesis in the context of our existing and future mouse models of neurofibroma and MPNST formation.

Finally, over the past several months, we have begun a differential cDNA cloning project in order to try to isolate genes that are specifically up-regulated in *Nf1*-/- MEFs that survive in the absence of serum growth factors. Subtractive hybridization is being performed between a "driver" population of composed of a mixture of cDNA derived from wild-type MEFs grown in 0.1% serum (these cells are alive but quiescent) and cDNA derived from serum-starved wild-type MEFs (at a time when a significant portion of the cells were undergoing apoptosis) and a "tester" population composed of cDNA derived from *Nf1*-/- cells in serum-starved conditions (which survive inappropriately). By interchanging the tester and driver populations it will also be possible to analyze genes that are downregulated in *Nf1*-deficient cells under these conditions. The isolation of genes from this screen should help to elucidate the pathway between cytoplasmic signaling regulated by neurofibromin and nuclear gene expression. One also anticipates that the

expression of a subset of these genes will be relevant to the development of neurofibromas and the other lesions associated with NF1.

3. Screen for genes modifying *Nf1* or *Nf2* in mice. Evidence from studies of variable expressivity in NF1 suggests that modifier genes affect the severity of the disease in humans (13). Mouse models of NF1 and NF2 provide a powerful tool for identifying modifier genes in different inbred strains because large numbers of genetically identical individuals can be bred for analysis. As outlined in the original proposal, we are generating inbred strains of mice carrying pairwise mutations of *Nf1*, *Nf2*, and *p53* in cis on chromosome 11 to map modifier genes. Since the last progress report, we have completed construction of several inbred strains. *Nf1;p53* cis heterozygotes (NPcis) have been generated on the C57BL/6J (B6), BALB/cJ, and 129S4/SvJae backgrounds, and *Nf2;p53* cis heterozygotes have been generated on the B6 background. We have begun to generate *Nf1;Nf2* heterozygotes in trans, for generation of *Nf1;Nf2* cis mice, on a B6 background. Finally, we have generated mice carrying a mutation in *Nf2* in trans to cis mutations of *Nf1;p53* on a B6 background. These mice have been crossed to wild-type B6 mice and we are genotyping progeny to identify recombination events (in approximately 20% of progeny) between *Nf2* and *p53* to generate *Nf1;Nf2;p53* triple cis mice.

To compare the tumor phenotype between different inbred strains, we have aged 30 NPcis mice on an inbred BALB/cJ background and 36 NPcis mice on an inbred B6 background. Of these mice, 90% of BALB/c mice and 83% of B6 mice have been sacrificed due to tumor burden. Based on these mice, there appears to be no difference in the survival curves for NPcis mice on these two strains. We are currently analyzing the tumor spectra of these two groups to determine if there are differences between the two strains.

As described in the previous annual report, we have been using NPcis mice on an enriched B6 background (not fully inbred) to identify promising inbred strain combinations for mapping of modifier genes. We have crossed these NPcis enriched B6 mice to 7 tester strains [A/J (A), CBA/J (CB), C3H/HeJ (H), DBA/2J (D2), LP/J, SJL/J (S), and CAST/EiJ (CA)]. Because of difficulties in breeding LP/J mice, analysis of this strain has been discontinued. Survival analysis has been completed for (A X B6)F1s (N=23), (CB X B6)F1s (N=27), (H X B6)F1s (N=27), (D2 X B6)F1s (N=22), and (S X B6)F1s (N=36). Only one mouse remains alive in the (CA X B6)F1 analysis (N=32). We are currently completing analysis of the histology of these strains to examine changes in the tumor spectra. All F1 strains are being compared to the enriched B6 strain (backcross generations 7 to 9; N=45). Both (A X B6)F1 and (CA X B6)F1 mice show a delay in the onset of tumor development compared to B6 (Fig. 5). In addition, (H X B6)F1 and (S X B6)F1 mice show acceleration of tumor development relative to B6 (Fig. 5). Because the differences between (A X B6)F1 vs. B6 and (CA X B6)F1 vs. B6 are at younger ages, we can determine differences in backcross progeny sooner in these strain combinations. We have chosen to focus on the (A X B6) X B6 and (CA X B6) X B6 strain combinations for mapping of backcross progeny. We have begun generating these backcross progeny for analysis of their tumor latency and spectra.

Preliminary data on the tumor spectra of B6, (H X B6)F1, (CA X B6)F1, and (S X B6)F1 show differences in tumor spectra between the different strains. We have compared the numbers of tumors for each strain for the major tumor types seen: soft-tissue sarcomas (including rhabdomyosarcomas, fibrosarcomas, MPNSTs), CNS tumors (see below), lymphoma, leukemia, pheochromocytomas, histiocytic sarcomas, and lyomyosarcomas. The most significant differences are seen between the B6 strain (N=36) and the (CA X B6)F1 strain (N=22). (CA X B6)F1 mice show a significant increase in leukemia ($P<0.05$, χ^2 test) and histiocytic sarcoma ($P<0.001$). (S X B6)F1 mice (N=34) also show a suggestive reduction in lymphoma ($0.01>P>0.05$). These results on hematopoetic cancer are particularly interesting given that children with NF1 are at an increased risk for developing myeloid leukemia. Identification of modifiers in mice may give insight into treatment of leukemia in human NF1 patients. In addition to effects on hematopoetic cancers, there are differences in (CA X B6)F1 vs. B6 and (S X B6)F1 vs. B6 in the development of brain tumors (see below). There were no significant differences between (H X B6)F1 mice (N=25) and B6 mice.

A mouse model of glioblastoma. The most frequent tumor type in NPcis mice on a B6 background are CNS lesions, affecting 77% of mice analyzed thus far (N=22). These lesions range from the presence of aberrant nuclei in the brain or spine to large, focal tumors (in 64% of B6 mice) causing ataxia or paralysis in the animal. We have compared these tumors to human astrocytomas by examination of hematoxylin/eosin stained sections and by immunohistochemistry with an astrocyte marker (glial fibrillary acidic protein, GFAP) and a neural marker (synaptophysin). These lesions show many of the histological hallmarks of grade II through grade IV (WHO criteria) astrocytomas, including aberrant nuclei, mitotic figures, formation of secondary structures around neurons, blood vessels, and the subpial zone of the cortex, as well as giant cells. We have not observed angiogenesis or necrosis (diagnostic criteria for grade IV human astrocytomas) in NPcis mice on a B6 background; however, we do see evidence for angiogenesis or necrosis in NPcis mice on other backgrounds. At least 40% of the focal tumors analyzed (N=10, B6) show positive tumor staining for the astrocyte marker GFAP, whereas none of the tumors show positive staining for the neural marker synaptophysin. The presence of astrocytomas in these mice is particularly interesting given that human NF1 patients are at an increased risk for developing malignant astrocytoma and glioblastoma, and loss of p53 has been identified as an early step in the development of a subset of spontaneous human glioblastomas.

We have compared the development of these astrocytomas on three different F1 strains thus far ((H X B6)F1, (CA X B6)F1, and (S X B6)F1). Of the (H X B6)F1 mice, 75% (N=24) develop some form of brain lesion, and 58% develop frank brain tumors. This is not significantly different from the B6 strain. Although (CA X B6)F1 and (S X B6)F1 mice do not show significantly different numbers of mice developing brain lesions (53% for (CA X B6)F1, N=19; and 55% for (S X B6)F1, N=33), (S X B6)F1 mice show significantly fewer focal tumors (24%, $P<0.001$), whereas (CA X B6)F1 mice show a suggestive reduction in focal tumors (47%, $0.1>P>0.05$). In addition, on all three F1 strains we see evidence for either necrosis or angiogenesis by histological criteria, suggesting that tumors progress to glioblastoma multiforms in these strains. Figure 6

shows an example of glioblastoma multiform in an NPcis mouse on the (H X B6)F1 background. This tumor contained many mitotic figures, giant cells, microscopic foci of necrosis, and was strongly immunopositive for GFAP.

4. Characterizing *Nf2* deficiency in adult chimeras and MEFs. As described in the previous annual report, we have observed that Rac1 induces the phosphorylation of the *Nf2* gene product, merlin. In addition, we have shown that *Nf2*-/- MEFs (isolated from *Nf2*+/+:*Nf2*-/- chimeras) have increased motility compared to wild-type MEFs, which would be consistent with an upregulation of Rac1 signaling in the cells. Over the past year, we have approached the functional interaction between Rac1 and merlin in two ways. First, in a close collaboration with the laboratory of my former post-doctoral fellow Andrea McClatchey (MGH), we have shown that various markers of Rac1 signaling are indeed elevated in *Nf2*-/- cells and that this effect can be suppressed through the transfection of wild-type *Nf2* expression vectors (data not shown). A manuscript describing these results will be submitted shortly. My intention is to leave much of the further characterization of the signal transduction pathway between Rac1 and merlin to Dr. McClatchey, with the exception of the isolation of the merlin kinase described below.

5. Continued investigation of merlin function.. Despite the increasing interest in the study of the *Nf2* gene and its product, merlin, its functions have remained elusive. One approach to the elucidation of this functions is the study of *Nf2* and merlin regulation. We had initially examined merlin protein levels and phosphorylation before and after various forms of cellular stress. We have shown that under conditions of confluency or serum starvation there is a decrease in the levels of both phosphorylated and unphosphorylated forms of merlin. In addition, loss of adhesion leads to a dephosphorylation of merlin, which is reversed when cells adhere (14). Thus, various stimuli like adhesion and growth conditions affect merlin in a post-translational manner that, most probably, serve to regulate its function. As it would seem that phosphorylation of merlin might have a central role in its regulation, discovering the identity of the relevant kinase(s) should shed light on the pathways involved in the regulation of merlin. Towards this aim we have set out to identify the kinase, employing a biochemical approach of protein purification.

At the heart of any such purification is an assay to determine the activity of the desired protein. Hence, our first step was to establish such an assay. This assay requires abundant amounts of substrate to be used in an *in vitro* kinase assay. As merlin is poorly expressed in bacterial cells (our unpublished data), the approach chosen was to create a pseudo-substrate in which the phosphorylation site of merlin was fused to GST. When expressed in bacteria this substrate yielded protein in milligram quantities. The substrate was then tested in kinase assays with protein extracts from NIH 3T3 cells, treated as described (14). As a negative control, a mutated form of the substrate was used in which serine 518 of merlin was changed to alanine. As shown in Figure 7, the phosphorylation of the protein is specific, as the mutated form of the substrate was not phosphorylated. Equal amounts of substrate were used in the assays. Switching of serine 518 to threonine 518 still resulted in the phosphorylation of a substrate, which serves as an additional indication of the specificity. With a specific assay in hand we further optimized the assay conditions, such as substrate and extract levels and reaction buffer composition.

Employing the *in vitro* kinase assay to assess activity, we have begun to test various approaches to purify the kinase. As a first step, we established conditions for ammonium sulfate precipitation of the kinase. This step results in 3.5-fold enrichment of the kinase activity, with a recovery of over 90%. The next steps tested were chromatographic in nature. For hydrophobic interaction chromatography, the appropriate column was selected and fractionation conditions determined. As a second step after salt precipitation, this approach yields a 10-fold enrichment with a recovery of about 70%. For ion-exchange chromatography and size-exclusion, the conditions and appropriate columns were determined. We are currently in the process of optimizing these steps and determining their effectiveness in series. In addition, we are in the early stages of testing other methods of separation. These include separation on hydroxylapatite and affinity chromatography by binding to nucleotides and nucleotide-like compounds such as dye-ligands.

Many studies of the ERM proteins have suggested that they are key components of the apical cytoskeleton in polarized epithelial cells. The demonstration of a critical role for ezrin in migration and tubulogenesis downstream of the HGF receptor in the porcine kidney epithelial cell line LLC-PK1 (15) prompted us to examine merlin function in these cells. In order to examine the effects of loss of merlin function in these cells, we set out to create a dominant negative allele of merlin. Several groups have very recently reported the creation of such molecules (16). In Drosophila, similar mutations in the N-terminal FERM domain of merlin have been made (17). A deletion or alanine-substitution of a seven amino acid stretch in merlin (a region designated "blue box," which is completely conserved between fly and mammalian merlin, but not in the ERMs) resulted in a dominant negative allele. When these blue box mutants were overexpressed in the fly, the same phenotype was observed as with somatic loss of function of merlin -- overproliferation of wing epithelial cells(17).

Because of the complete conservation of this seven amino region between Drosophila and mammalian merlin, we set about constructing the alanine substitution blue box mutant in the context of full-length mouse merlin (designated "FDN": Fehon dominant negative). To examine the effects of this allele in LLC-PK1 epithelial cells, we generated stable cell lines expressing HA-tagged FDN as well as HA-wild-type merlin, and HA-ezrin. Initial observations have suggested that cell lines that overexpressed FDN-merlin exhibited many characteristics of *Nf2*-deficient fibroblasts: they seemed to grow to a higher density and continued to grow on top of one another, and they continued to proliferate in the absence of serum. Further basic characterization of this cell line in order to assess the dominant-negative allele is ongoing, including localization studies, HGF-dependent motility and tubule formation assays, ezrin phosphorylation and binding status, and Rac activation status. It is notable that stable overexpression of wild-type merlin is not well tolerated in these cells; the percentage of cells expressing HA-wildtype merlin in the "stable population" declines with every passage even in the presence of antibiotic selection.

Consequently, we are continuing efforts to create a conditional system in which we can control expression of wild-type merlin isoforms as well as other merlin mutant constructs, including FDN-merlin. Such a system will allow us to study better the role of

merlin in the LLC-PK1 epithelial cells. Furthermore, we can use these reagents in order to reintroduce merlin constructs into *Nf2* -/- tumor cell lines to study the role of merlin isoforms (and other constructs) in tumorigenesis. Introduction of phospho-mutants as well as N/C-terminal domains of merlin may help to elucidate some structure-function data including the role that Rac-mediated phosphorylation at amino acid 518 plays on merlin's activity in the cell. Previous attempts at using the tetracycline-inducible system (described in our previous update, 1998) have been unsuccessful. Consequently, the preparation of reagents using the ecdysone conditional expression system is currently underway.

Finally, in a recent study, Gronholm and colleagues (18) reported that merlin can interact with ezrin. Using co-immunoprecipitation studies combined with a yeast two-hybrid system, blot overlay and affinity precipitation, they were able to study this interaction as well as map the association domains (18). Because ezrin is known to promote cell survival, and merlin is described as a tumor suppressor, we set about examining possible functional and potentially physiologically relevant consequences of merlin-ezrin interactions. The current project investigates the role of merlin interactions with ezrin and their potential effect on ezrin's recently reported interactions with p85α and promotion of cell survival through the AKT/PKB pathway (19). Experiments, including co-immunoprecipitation and affinity precipitation after *in vitro* transcription/translation as well as assays providing functional readouts for AKT and/or PI3K activity, are currently underway. The authors' original observations have been verified. We hope that these studies will lead us to a better understanding of merlin's role as a tumor suppressor. Such investigations may also reveal new binding partners for merlin that contribute to its function in the cell.

6. Therapeutic evaluation of farnesyltransferase inhibitors. A long term goal of the development of animal models of NF1 and NF2 is to use these animals in the evaluation of potential therapies for these diseases. Presently, one class of compounds, the farnesyltransferase inhibitors directed against the RAS signaling pathway, are logical candidates in the treatment of tumors with *NF1* mutations. In time other compounds will be developed with potential utility in the treatment of NF1 or NF2, which can be considered for use in these mice.

As described in the previous annual report, we have received from Merck Pharmaceuticals 1 gram of their farnesyltransferase inhibitor L-744,832, which has been used to treat RAS-dependent tumors in mice (20, 21). Given the uncertainty in the supply of this compound in the future, we have been very cautious about what animal model to use it in and under what specific conditions. Most recently, given the development of an *Nf1*-deficient brain tumor model (see above), we have decided to apply L-744,832 to it. These experiments will be underway shortly. Discussions continue with Merck and other pharmaceutical companies about gaining access to other agents that are targeted to the RAS pathway.

Conclusions

During the past year of funding, we have continued to make good progress toward our goals. In particular, the publication of the *Nf1* chimera model and the *Nf1/p53* model represent very important advances in this field. The genetic studies on the *Nf1/p53* model have produced interesting strain-specific differences in phenotype, which will be followed up over the next year, and the discovery of a brain tumor phenotype in these animals has important implications for NF1 and for the study of brain tumors more generally. Our work on the basic cell biological and biochemical pathways that regulate or are regulated by neurofibromin and merlin has accelerated over the past year, and we have discovered regulatory pathways that impinge on the normal gene products and that are affected through their mutation. The continuation of these studies is critical for understanding the normal function of the NF1 and NF2 tumor suppressor genes and the consequences of their loss in the course of disease development.

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Figure Legends

Figure 1. Neurofibromin is rapidly degraded in response to serum. NIH 3T3 cells were serum starved for 24 hours and treated with serum for increasing lengths of time. Western blots of total cell lysates were probed with a C-terminal (left) or N-terminal (right) neurofibromin antibody.

Figure 2. Neurofibromin is regulated by the proteasome. (a) NIH 3T3 cells were serum starved for 24 hours and treated with the proteasome inhibitors LLNL or lacticystin for 6 hours as specified. Cells were then stimulated with EGF, PDGF or LPA for 15 minutes or as labeled. Western blots of total cell lysates were probed with a C-terminal neurofibromin antibody. (b) A plasmid encoding a myc and his-tagged ubiquitin molecule was transfected into NIH3T3 cells. Cycling cells were treated with LLNL, lysed, and his-tagged proteins were purified using a nickel column. These proteins were then separated by gel electrophoresis and a western blot was probed with the C-terminal neurofibromin antibody.

Figure 3. Neurofibromin degradation is dependent on protein kinase C activity. (a) NIH 3T3 cells were serum starved for 24 hours and treated with the protein kinase C inhibitors bisindolylmaleimide I or II as specified. Cells were then stimulated with LPA for increasing lengths of time and western blots of total cell lysates were probed with a C-terminal neurofibromin antibody. (b) A control plasmid or plasmids encoding activated PKC isozymes were co-transfected with limiting concentrations of an EGFP-expressing plasmid into NIH 3T3 cells. Transfected cells were sorted by FACS, by virtue of GFP expression, and total cell lysates were prepared and normalized. Western blots were probed with the C-terminal neurofibromin antibody.

Figure 4. ERK activity is sustained in *Nf1*-/- MEFs in response to low concentrations of serum. *Nf1* +/+ and *Nf1*-/- cells were serum starved for 24 hours and treated with various concentrations of serum for increasing lengths of time. Western blots of total cell lysates were probed with a phospho-specific ERK antibody.

Figure 5. Effects of Genetic Background on Survival of NPcis mice. (a) compares the survival curve of NPcis mice on a B6 background to F1 NPcis mice on two different strain combinations [(A X B6)F1 (A) and (CA X B6)F1 (CAST)]. These two F1 strains show a delay in onset of tumors by approximately 1 month, and also show a steeper survival curve than the parental B6 line. Panel (b) compares the survival curves of NPcis mice on the parental B6 background and the F1 strains (H X B6)F1 (C3H) and (S X B6)F1 (SJL). These two F1 strains show similar onset of tumor formation, but an increased rate of tumor development, leading to a steeper survival curve.

Figure 6: Glioblastoma Multiforme in NPcis Mice. The figure shows a brain tumor found in an NPcis mouse on the (H X B6)F1 background. The tumor fits the histological criteria of a WHO grade IV glioblastoma multiforme. In the left panel the tumor is taking up the majority of the brain space, compressing the cerebellum (C). The pituitary (P) is labeled for reference. The main tumor mass (TM) is densely cellular, with microscopic foci of necrosis. At the ventral and posterior boundaries the tumor is showing an infiltrative growth pattern (IG) typical of many of the tumors we see. The right panel shows a 100-fold magnification of the left panel. Nuclei show aberrant morphology. Arrows indicate mitotic figures and arrowheads indicate regions of necrosis, indicative of a grade IV tumor.

Figure 7. Specific phosphorylation of a pseudo-substrate by merlin-kinase activity. Various substrates were used in an in-vitro kinase assay with NIH3T3 total protein extracts and [γ-^{32}P]-ATP. S518 is the substrate unaltered with serine at position 518, A518 has alanine at 518 and T518 has a threonine.

Figure 1

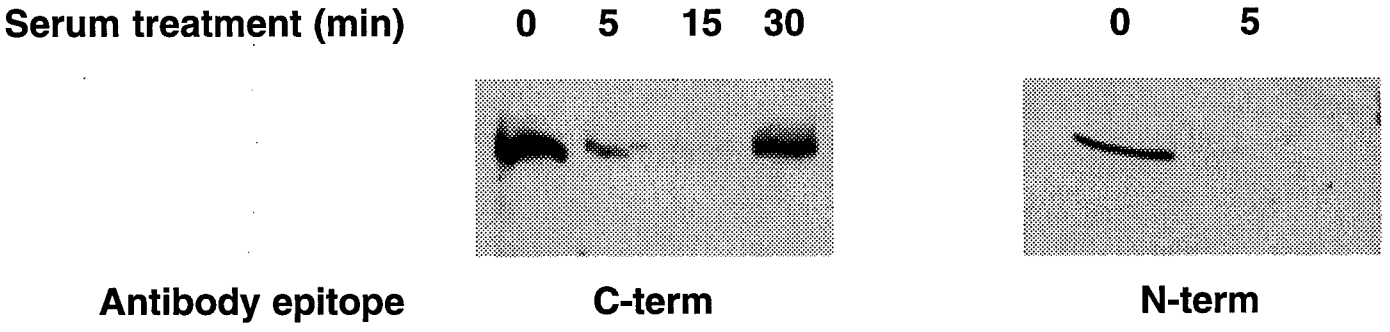
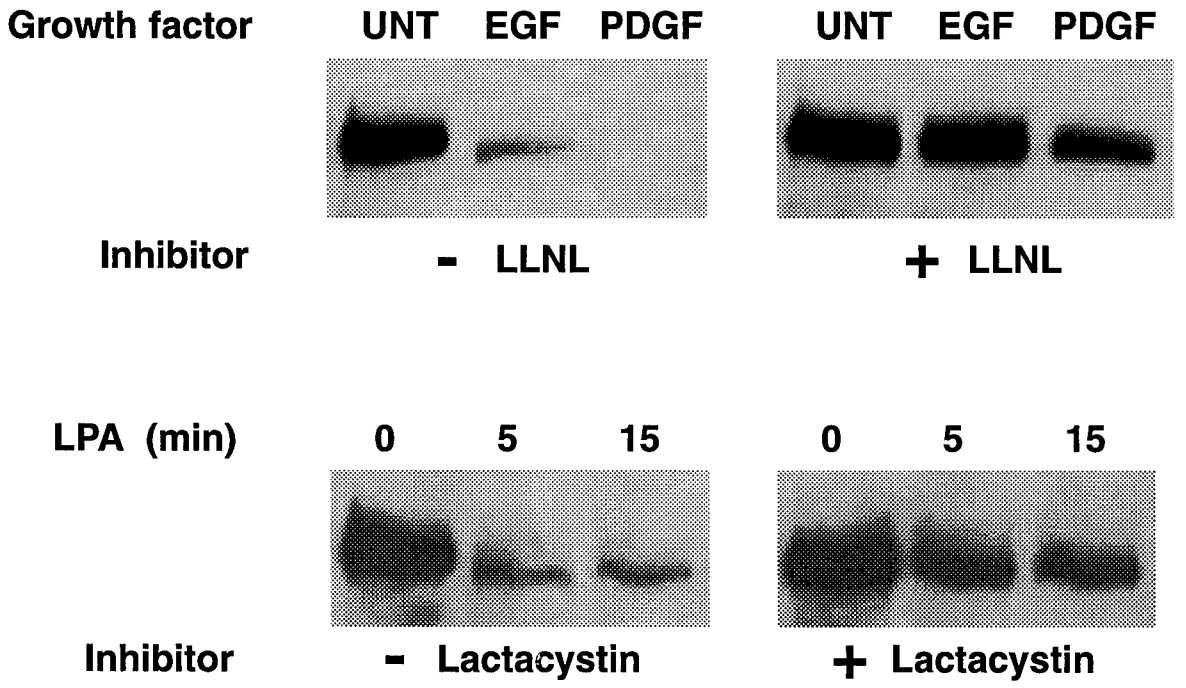

Serum treatment (min)
0
5
15
30
0
5
Antibody epitope
C-term
N-term

Figure 2

A


Growth factor
UNT EGF PDGF
UNT EGF PDGF
Inhibitor
- LLNL
+ LLNL
LPA (min)
0 5 15
0 5 15
Inhibitor
- Lactacystin
+ Lactacystin

B

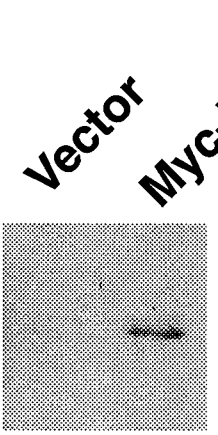
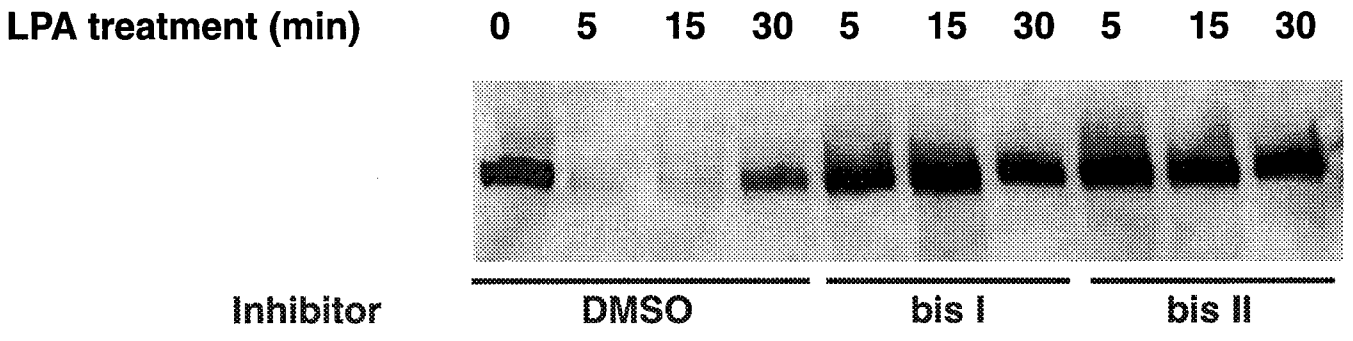

Vector
Myc-Ub-His

Figure 3

A


LPA treatment (min)
0 5 15 30 5 15 30 5 15 30
Inhibitor
DMSO
bis I
bis II

B

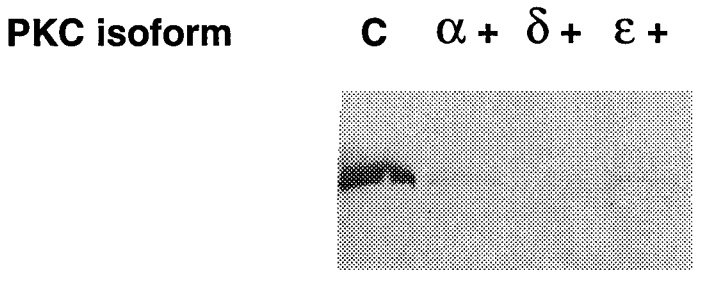

PKC isoform
C α + δ + ε +

Figure 4

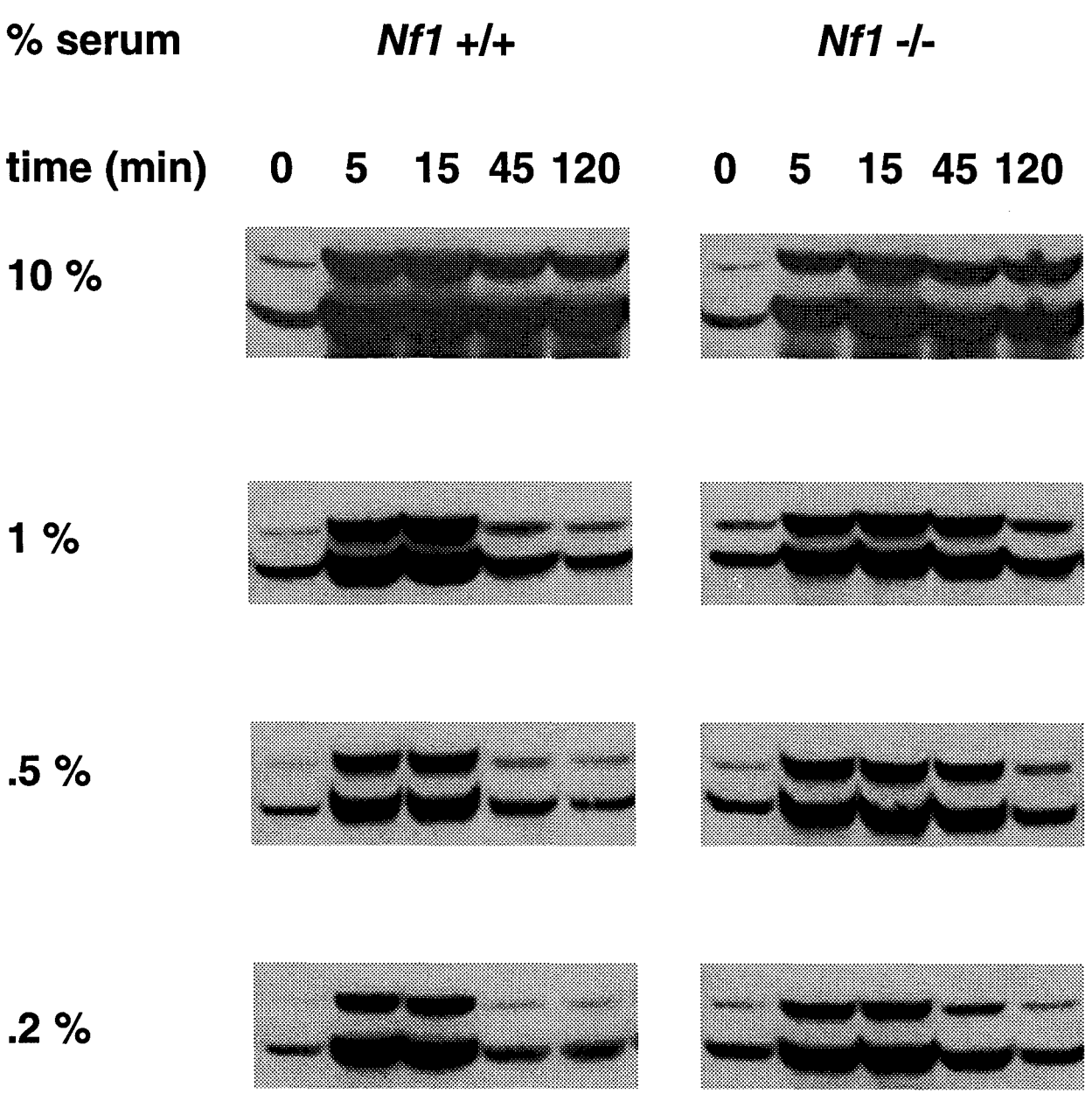


Figure 5

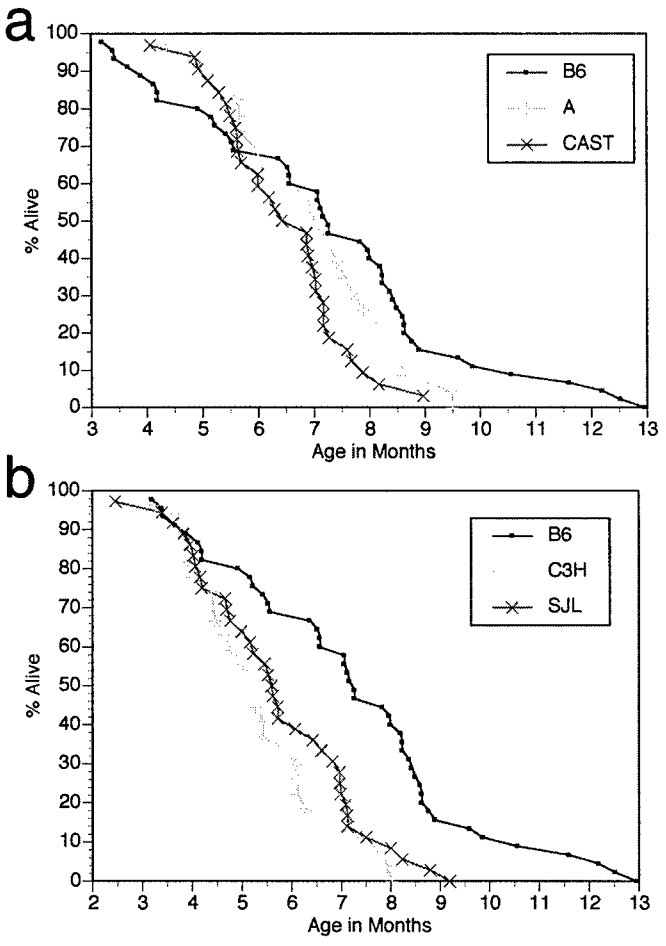


Figure 6

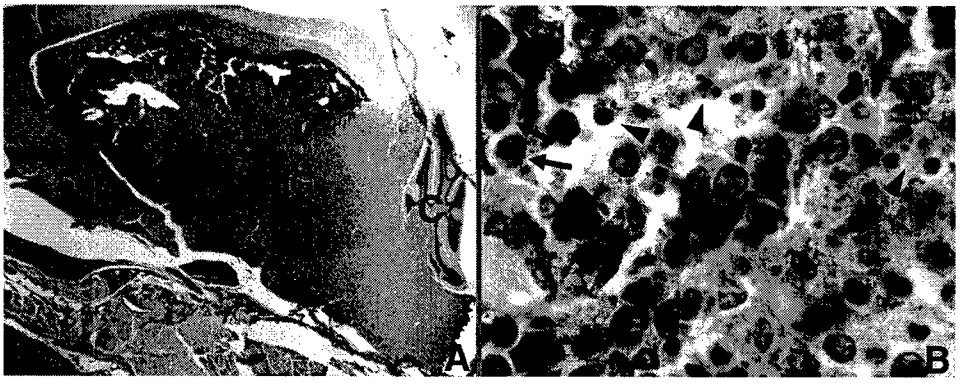
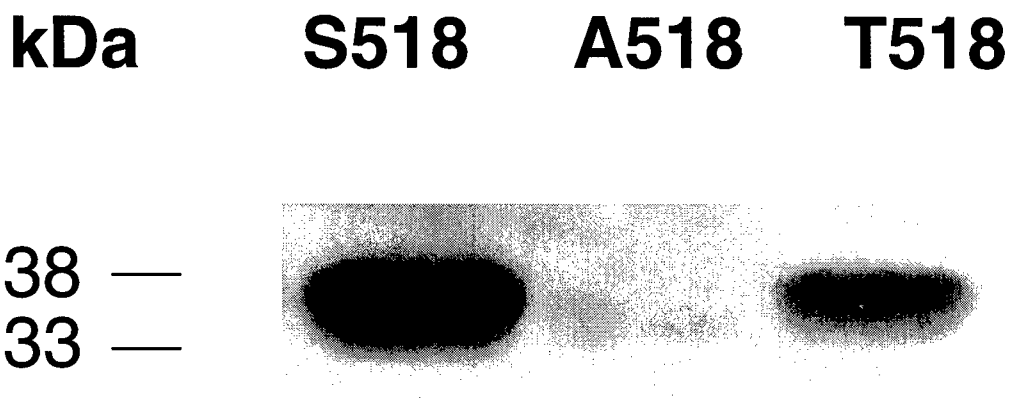


Figure 7



DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

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SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to the enclosed list of technical documents. Request the limited distribution statement assigned to the documents listed be changed to "Approved for public release; distribution unlimited." These documents should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

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Deputy Chief of Staff for
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